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Biological monitoring in workers in a nitrobenzene reduction plant: haemoglobin versus serum albumin adducts

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Abstract The high priority of monitoring workers exposed to nitrobenzene is a consequence of clear findings of experimental carcinogenicity of nitrobenzene and the associated evaluations by the International Agency for Research on Cancer. Eighty male employees of a nitrobenzene reduction plant, with potential skin contact with nitrobenzene and aniline, participated in a current medical surveillance programme. Blood samples were routinely taken and analysed for aniline, 4-aminodiphenyl (4-ADP) and benzidine adducts of haemoglobin (Hb) and human serum albumin (HSA). Also, levels of methaemoglobin (Met-Hb) and of carbon monoxide haemoglobin (CO-Hb) were monitored. Effects of smoking were straightforward. Using the rank sum test of Wilcoxon, we found that very clear-cut and statistically significant smoking effects (about 3-fold increases) were apparent on CO-Hb ($P = 0.00085$) and on the Hb adduct of 4-ADP ($P = 0.0006$). The mean aniline-Hb adduct level in smokers was 1.5 times higher than in non-smokers; the significance ($P = 0.05375$) was close to the 5% level. The strongest correlation was evident between the Hb and HSA adducts of aniline ($r_s = 0.846$). Less pronounced correlations (but with P values < 0.02) appeared between aniline-Hb and 4-ADP-Hb adducts ($r_s = 0.388$), between 4-ADP and 4-ADP-HSA adducts ($r_s = 0.373$), and between 4-ADP-Hb and aniline-HSA adducts ($r_s = 0.275$). In view of the proposal for additional use of the aniline-HSA adduct for biological monitoring, particularly in cases of acute overexposures or poisonings, the strong correlation of the Hb and HSA conjugates is noteworthy; the ratio aniline-HSA:aniline-Hb was 1:42 for the entire cohort.

Key words Nitrobenzene · Aniline · Biological monitoring · Haemoglobin adducts · Serum albumin adducts

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Introduction

As aromatic nitro-compounds and aromatic amines have a high potential for skin penetration (Levillain et al. 1998), relevant industrial exposures to nitrobenzene and aniline occur by skin contact (Pendergrass 1994). For such compounds, biological monitoring strategies are being officially recommended (Bundesminister für Arbeit und Sozialordnung 1996).

Based on animal experimentation (Albrecht and Neumann 1985) and on field observations in exposed industrial workers (Lewalter and Korallus 1985) the concept has been promoted to use hydrolysable haemoglobin (Hb) adducts of aromatic amines as dosimeters of exposures and markers of metabolism of aromatic amines and associated nitro-compounds (Sepai and Sabbioni 1996).

The need of such strategies for the surveillance of workers specifically exposed to nitrobenzene has been much accentuated by clear findings of experimental carcinogenicity of nitrobenzene in B6C3F1 mice, Fischer 344 rats and CD rats (Cattley et al. 1994) which also gave rise to a classification of nitrobenzene by the International Agency for Research on Cancer (IARC 1996) as "possibly carcinogenic to humans (Group 2B)".

The redox couple phenylhydroxylamine/nitrosobenzene is critical for the systemic toxicity and carcinogenicity of both aniline and nitrobenzene (Holder 1999).

Table 1 Biological monitoring data (Met-Hb, CO-Hb, Hb and HSA adducts) of workers employed at the LS-T2 nitrobenzene reduction plant [*N* non-smoker, *S* smoker, *OS* occasional smoker, *n.d.* not detectable, *n.b.* no blood available for determination, – (CO-Hb) no result available]

Code no.	Met-Hb (%)	CO-Hb (%)	Smoking habits	Aniline-Hb (ng/l blood)	4-ADP-Hb (ng/l blood)	Aniline-HSA (ng/l blood)	4-ADP-HSA (ng/l blood)
2	0.4	0.6	N	1,200	3.6	< 100	0.1
4	0.8	5.1		1,800	14	< 100	0.2
5	n.d.	–		3,300	2.6	200	0.4
6	0.5	0.5		1,300	2.5	< 100	0.3
7	0.8	5.5		700	12.1	< 100	0.3
8	1.4	–	N	6,400	7.5	250	< 0.1
9	0.7	4.7	S	6,500	24	140	0.2
10	0.7	–		2,300	7.8	110	< 0.1
11	0.8	7.1	S	2,900	11.1	< 100	0.1
13	0.8	0.9		1,300	3.1	< 100	< 0.1
14	0.4	1.3	N	470	2	< 100	< 0.1
16	1	4.9	S	1,200	11.4	< 100	< 0.1
17	0.2	6	S	2,100	9.3	< 100	0.1
19	0.7	5.5		n.b.	n.b.	n.b.	n.b.
22	0.8	0.8	N	13,700	2	250	< 0.1
23	0.4	1.1	N	1,300	2.2	< 100	< 0.1
24	1.3	0.5	N	4,900	1.6	140	0.1
27	0.8	–		2,200	19	< 100	0.4
28	0.7	4.2		1,700	12.6	< 100	< 0.1
29	n.d.	–		1,600	1.3	< 100	< 0.1
30	1	–		10,800	2.7	280	< 0.1
32	0.6	3.4		3,100	11.2	< 100	0.3
33	0.4	1.2		2,900	2.4	< 100	< 0.1
34	0.2	1.5		4,400	2.1	190	0.1
35	0.8	5		4,600	11.1	160	0.2
36	0.8	0.4	N	430	1.7	< 100	< 0.1
48	0.4	–	S	3,200	14.6	< 100	< 0.1
49	0.5	0.8		2,800	1.7	100	< 0.1
52	n.d.	–	S	5,400	13.9	< 100	< 0.1
54	n.d.	–		6,200	5.2	210	0.2
55	0.4	5.2		13,900	9.7	250	0.2
56	0.6	4.8		7,500	13.1	120	0.1
57	0.8	1.9	OS	12,400	4.3	150	0.4
58	0.8	5.3		n.d.	n.d.	n.d.	n.d.
59	0.6	6.3		16,700	17	350	0.2
61	n.d.	–		1,100	1.9	< 100	< 0.1
62	1.1	4.1		12,500	17	320	< 0.1
63	n.d.	...		3,600	3.2	< 100	< 0.1
64	0.6	0.4		n.b.	n.b.	n.b.	n.b.
65	n.d.	...		n.d.	n.d.	n.d.	n.d.
66	1.5	5.5		25,500	14.3	650	< 0.1
67	0.5	–		3,300	2.5	100	< 0.1
69	0.5	5.5		9,100	17.8	140	0.3
71	0.1	1.9	N	1,900	2.2	< 100	< 0.1
72	1.1	–		1,700	3.7	< 100	0.1
73	n.d.	–	S	3,900	21.8	110	0.4
74	n.d.	–		510	10.3	< 100	0.3
75	1	–		4,600	3.3	100	< 0.1
76	0.7	7.5	S	9,900	10.7	200	0.1
77	0.5	0.6	S	5,700	5.8	< 100	< 0.1
78	n.d.	–	N	6,100	4.6	310	0.2
79	0.4	3	S	18,200	8.6	320	0.2
80	n.d.	–		3,400	2.3	250	0.2
82	n.d.	–	N	6,500	14.9	230	0.3
83	n.d.	–	S	7,900	13.1	240	0.3
84	0.4	1.1		2,900	2.1	< 100	< 0.1
86	0.5	1.3		1,300	2.1	< 100	0.1
87	0.7	0.7		900	2.6	< 100	0.4
88	0.7	0.5	N	1,700	3.6	< 100	< 0.1
89	0.7	4.9	S	2,900	10.7	< 100	< 0.1
90	n.d.	–	S	7,700	11.6	300	0.4
91	0.5	6.4		10,800	11.8	230	0.2
92	0.8	0.5	S	8,300	4.1	350	< 0.1
93	0.7	6.8		11,700	10.8	200	< 0.1

Table 1 (Continued)

Code no.	Met-Hb (%)	CO-Hb (%)	Smoking habits	Aniline-Hb (ng/l blood)	4-ADP-Hb (ng/l blood)	Aniline-HSA (ng/l blood)	4-ADP-HSA (ng/l blood)
94	0.6	6.2		23,500	13	1100	0.3
95	n.d.	–		11,800	13.3	470	0.4
96	0.8	4.1	S	16,500	12.2	260	< 0.1
97	n.d.	–	N	7,600	4.5	310	0.5
98	0.6	1.3	N	3,800	1.3	< 100	< 0.1
99	0.9	0.7		12,100	2.3	330	0.4
100	0.5	2		9,000	5.2	210	< 0.1
101	0.5	2.3	S	13,200	3.5	220	< 0.1
102	0.8	0.7	N	27,000	18.4	430	< 0.1
106	0.6	1.7	N	6,300	1.9	260	< 0.1
107	1	4.5	S	9,700	6.4	160	< 0.1
112	1.1	–		18,500	3	250	< 0.1
113	0.5	1.5		3,800	1.9	140	< 0.1
114	1.3	4		n.b.	n.b.	n.b.	n.b.
115	n.d.	–		2,100	12.1	130	0.6
116	0.3	–		7,600	3.2	< 100	0.1

Aromatic nitroso-compounds are the biologically relevant metabolites which are reactive with proteins (Zwirner-Baier et al. 1994).

As an orientation for the biological monitoring of workers exposed to nitrobenzene and/or aniline the Deutsche Forschungsgemeinschaft has established biological tolerance (BAT) values of 100 µg aniline, released from the aniline-Hb conjugate per litre whole blood (Greim and Lehnert 1995). Discussions on further refinements of the protein adduct monitoring in exposed workers were held following casuistic observations of human accidental industrial poisonings (Lewalter and Neumann 1998); it was suggested to make diagnostic use of the widely differing life-spans of blood proteins. Specifically, the long life-span of Hb (equivalent to that of erythrocytes of ≈120 days) contrasts to the relatively short half-life of human serum albumin (HSA; t1/2 ≈19 days). The integration of analysis of HSA adducts and calculation of time-dependent Hb/HSA adduct ratios was viewed as a powerful tool in the surveillance of workers after acute incidental overexposures to nitrobenzene and/or aniline.

However, comparative data of HSA and Hb adducts in workers exposed under current industrial conditions have not been published so far. The present communication reports on such data in 80 workers employed in an industrial nitrobenzene reduction plant. Under current medical surveillance programmes comparative Hb and HSA adduct data and data on methaemoglobin (Met-Hb) and carbon monoxide haemoglobin (CO-Hb) levels were accessible.

Materials and methods

Eighty male employees of a nitrobenzene reduction plant, with potential skin contact to nitrobenzene and aniline, participated in a current medical surveillance programme. A typical condition for this type of workplace is that exposures occur via skin contact and through the airborne route; airborne exposures are normally low. Aniline concentrations in the workplace air at the plant were measured periodically between 1987 and 1999, resulting in a mean of 1.20 mg/m^3 (SD: 1.21 mg/m^3; MAK = 7.7 mg/m^3 as of 2000). In such a situation, valid exposure assessments can only be based on biological monitoring data, not on analysis of ambient air alone. Also, it is characteristic that quantities of dermal exposure are very variable with time and subject.

Blood samples were routinely taken and analysed for aniline, 4-aminodiphenyl (4-ADP) and benzidine adducts of Hb and HSA. Also, the levels of Met-Hb and CO-Hb were spectroscopically monitored. Due to organisational and personal matters, the current smoking status was not known for all of the persons. In the total cohort there were 80 observations of Met-Hb and CO-Hb and 33 observations (15 non-smokers, 18 smokers) of Met-Hb and CO-Hb in the subgroup in which the smoking status was known (one "occasional" smoker was classified as smoker). There were 77 observations of Hb and HSA adducts within the total cohort and 33 in the subcohort of known smoking status.

Analytical procedures

The methods applied to determine aniline conjugates to blood proteins and to determine Met-Hb and CO-Hb have been evaluated by the Working Group "Analyses of Hazardous Substances in Biological Materials" of the Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area of the Deutsche Forschungsgemeinschaft (Angerer and Schaller 1994).

The principles of biomonitoring of aniline and nitrobenzene exposures by analysis of the aniline-Hb adduct have been reviewed by Albrecht and Neumann (1985). A detailed working scheme for analysis of conjugates of aromatic amines to blood proteins, including the analytical calibration procedures, has been described by Lewalter and Korallus (1985). This particular procedure has been used in the current study.

In principle, globin is isolated from erythrocyte lysate. The conjugate arising from nitrobenzene or aniline to the SH-group of cysteine in globin is subjected to acid hydrolysis (HCl), and the resulting aniline is extracted. The quantitation is performed by gas chromatography, using a nitrogen-specific detector. The analytical detection limit amounted to 1 ng aniline or 4-ADP adducted to globin or HSA per litre of blood.

Statistical analysis

In order also to integrate "non-detectable" biological values, we examined effects of smoking using Wilcoxon's rank sum test. For the same reason, Spearman's correlation coefficients were calcu-

Table 2 Means and standard deviations (MMLE) of biomonitoring data in the entire cohort (Met-Hb and CO-Hb, $n = 79$; otherwise $n = 75$) and in the subcohorts of known non-smokers ($n = 15$) and smokers ($n = 18$)

Analyte	Entire cohort	Non-smokers	Smokers
Met-Hb (%)	0.455 ± 0.443	0.443 ± 0.354	0.478 ± 0.472
CO-Hb (%)	1.12 ± 2.16	0.663 ± 2.88	1.95 ± 0.557
Adducts			
Aniline-Hb (ng/l)	5180 ± 5192	4589 ± 4609	6872 ± 5003
4-ADP-Hb (ng/l)	6.39 ± 5.48	3.96 ± 5.17	10.3 ± 3.66
Aniline-HSA (ng/l)	121 ± 144	153 ± 106	140 ± 126
4-ADP-HSA (ng/l)	0.0968 ± 0.137	0.0306 ± 0.135	0.0926 ± 0.0978

Table 3 Correlation data between different biological monitoring parameters (entire cohort). Met-Hb and CO-Hb expressed in %, aniline and 4-ADP adducts to Hb and HSA in ng adduct/l blood

Correlation *P* value *N*	Met-Hb	CO-Hb	Aniline-Hb	4-ADP-Hb	Aniline-HSA	4-ADP-HSA
Met-Hb	1	0.39109 0.0003	0.17395 0.1303	0.07230 0.5320	0.10139 0.3803	− 0.28692 0.0114
	79	79	75	75	75	75
CO-Hb		1	0.18372 0.1097	0.22516 0.0490	0.04522 0.6962	− 0.06353 0.5831
		79	75	75	75	75
Aniline-Hb			1	0.38847 0.0005	0.84516 0.0001	0.09974 0.3881
			75	75	75	75
4-ADP-Hb				1	0.27482 0.0156	0.37342 0.0008
				75	75	5
Aniline-HSA					1	0.21207 0.0641
					75	75
4-ADP-HSA						1 75

Table 4 Correlation data between different biological monitoring parameters (subcohort of known non-smokers). Met-Hb and CO-Hb expressed in %, aniline and 4-ADP adducts to Hb and HSA in ng adduct/l blood, $n = 15$

Correlation *P* value	Met-Hb	CO-Hb	Aniline-Hb	4-ADP-Hb	Aniline-HSA	4-ADP-HSA
Met-Hb	1	0.03927 0.8895	0.06324 0.8228	−0.28443 0.3042	0.00191 0.9946	−0.55575 0.0315
CO-Hb		1	−0.26175 0.3460	−0.53214 0.0412	0.38799 0.1530	−0.64240 0.0098
Aniline-Hb			1	0.47270 0.0752	0.86193 0.0001	0.25121 0.3665
4-ADP-Hb				1	0.50588 0.0544	0.3665 0.1979
Aniline-HSA					1	0.35077 0.1999
4-ADP-HSA						1

lated (Hollander and Wolfe 1999). Both methods are based on the ranks of the observations, i.e., they use the relative position of the observation to the rest of the sample, e.g. lowest observation, instead of the observations themselves. Thus, it is possible to label "non-detectable" values as lowest values and to calculate the respective statistics on the entire database, i.e., "detectable" and "non-detectable" values. The calculated *P* values resemble the probabilities of the independence of the variables in question. Modified maximum likelihood estimates (MMLE) based on the censored sample were calculated according to Tiku et al. (1986) after square-root transformation of the data. As the first partial derivatives of the likelihood function, which are used for the usually applied maximum likelihood estimates, do not admit explicit solutions in the case of censored samples, MMLE are obtained by approximating terms depending on the lowest and/or highest measurable observations. The MMLE are asymptotically identical with the maximum likelihood estimates.

Results

The analytical data of the entire cohort are given in Table 1. As there was no occupational exposure to benzidine, the benzidine adduct levels, both to Hb and

Table 5 Correlation data between different biological monitoring parameters (subcohort of known smokers). Met-Hb and CO-Hb expressed in %, aniline and 4-ADP adducts to Hb and HSA in ng adduct/l blood, $n = 18$

Correlation *P* value	Met-Hb	CO-Hb	Aniline-Hb	4-ADP-Hb	Aniline-HSA	4-ADP-HSA
Met-Hb	1	0.59101 0.0098	0.10315 0.6838	− 0.39375 0.1059	0.00108 0.9966	− 0.40189 0.0983
CO-Hb		1	− 0.13936 0.58113	− 0.23957 0.3383	− 0.22680 0.3655	− 0.15689 0.5341
Aniline-Hb			1	− 0.37552 0.1246	0.81800 0.0001	0.13086 0.6048
4-ADP-Hb				1	− 0.25423 0.3087	0.24730 0.33225
Aniline-HSA					1	0.27291 0.2732
4-ADP-HSA						1

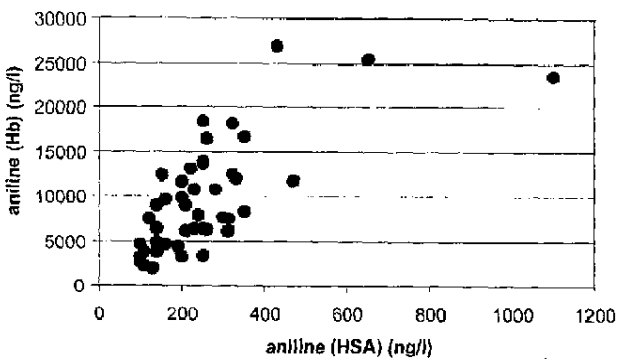

Fig. 1 Relationship between Hb and HSA adducts of aniline in the entire cohort of nitrobenzene reduction plant workers

HSA, were below the analytical limit of detection in all persons. These adducts were not further evaluated.

Considering the problem of values at and below the detection limit, we performed calculations of MMLE (Tiku et al. 1986) for means and variances. The group means and SDs for the entire cohort ($n = 79$ for Met-Hb and CO-Hb; $n = 75$ for adduct data) and for the subgroup of known smokers ($n = 18$) and non-smokers ($n = 15$) are displayed in Table 2. Using the rank sum test of Wilcoxon, we found that very clear-cut and statistically significant effects of smoking were apparent on CO-Hb ($P = 0.00085$) and on the Hb adduct of 4-ADP ($P = 0.0006$), confirming previous data reported by others (Bryant et al. 1987). The mean aniline-Hb adduct level in smokers was 1.5 times higher than in non-smokers; the significance ($P = 0.0538$) was close to the 5% level. No statistically significant differences between smokers and non-smokers were found for Met-Hb ($P = 0.876$), for aniline-HSA adducts ($P = 0.264$) and for HSA-4-ADP adducts ($P = 0.945$); the large variability of HSA-4-ADP adducts within the subgroups was particularly noteworthy.

Correlations between the biological parameters, using Spearman's rank sum correlation coefficients (v.s.), were calculated for the entire cohort (Table 3) and for the subcohorts of known non-smokers (Table 4) and smokers (Table 5). The tables show the coefficients of correlation and the *P* values. Numbers of observations (n) were 79 for Met-Hb and CO-Hb and 75 for the aniline and 4-ADP adducts in the total cohort, 15 for the subcohort of known non-smokers and 18 for that of smokers.

Not surprisingly, the strongest correlation was evident between the Hb and HSA adducts of aniline ($r_s = 0.846$). This connection is visualised in Fig. 1. Less pronounced correlations (but with *P* values < 0.02) appeared between aniline-Hb and 4-ADP-Hb adducts ($r_s = 0.388$), between 4-ADP and 4-ADP-HSA adducts ($r_s = 0.373$), and between 4-ADP-Hb and aniline-HSA adducts ($r_s = 0.275$).

Discussion

The high skin permeability of nitro- and amino-aromatic compounds calls for effective biological monitoring strategies (see Introduction section). For nitrobenzene, the Deutsche Forschungsgemeinschaft (Greim and Lehnert 1995) has established a BAT value; it is set as an "aniline content (released aniline) in the isolated haemoglobin adduct of 100 µg of aniline per litre of whole blood". As this value is orientated towards a maximally permissible Met-Hb level of 5%, observation of this parameter should "prevent damage attributable to a restriction of the oxygen transport capacity of the erythrocytes" (Greim and Lehnert 1995).

The legal definition of the BAT value in Germany is the "concentration of a chemical substance, or its metabolite, or the maximum permissible deviation from the norm of biological parameters induced by these substances which normally do not affect the health of employees" (Bundesminister für Arbeit und Sozialordnung 1999). The clear experimental carcinogenicity of nitrobenzene (IARC 1996) leads to the postulate of minimising exposures to this compound, to levels as low as reasonably achievable. Our data reveal current industrial exposure levels accompanied by aniline-Hb conjugate levels at about 1/20 of the BAT value. Higher adduct levels, in the order of the BAT value, may occur in cases of industrial intoxications (Lewalter and Neumann 1998).

In view of the proposal (Lewalter and Neumann 1998) for additional use of the aniline-HSA adduct for biological monitoring, particularly in cases of acute overexposure or poisoning, the strong correlation of the Hb and HSA conjugates is noteworthy. However, it must be noted that the adduct ratio observed here (aniline-HSA: aniline-Hb, 1: 42 for the entire cohort; Table 2, Fig. 1) is valid only for continuously low industrial exposure conditions; this ratio may even be reversed following an accidental short-term overexposure.

A critical point is the high proportion of censored data in the sample (e.g. up to 67% of 4-ADP-HSA adduct data in non-smokers below the detection limit of 0.1 ng/l blood). Generally, statistical methods based on ranks, such as the Spearman's rank correlation coefficient used here, represent an appropriate tool to overcome this problem. The Spearman's rank correlation coefficient requires only ordinally scaled observations, i.e. only the order, not the relative distances between the observations, must be known. More critical than this censoring problem is the occurrence of bindings, i.e. the existence of two or more observations with equal values. Whilst a modified Spearman's rank correlation coefficient also accounts for bindings, there are still no recommendations about the maximum size of the single binding groups. Concerning albumin adducts, there are binding groups of the censored data containing nearly or even more than 50% of the observations. Especially for small sample sizes, the related test statistics may fail to converge against the normal distribution. Despite this open question, we consider the use of the Spearman's rank correlation procedure as appropriate to deal with the censoring problem in general, and with the present case in particular.

The effects of smoking observed in the present study are straightforward. There is an approximately 3-fold elevation of CO-Hb and of the haemoglobin adduct of 4-ADP (Table 2). By contrast, the effect of smoking on the aniline adducts (both aniline-Hb and aniline-HSA) is only marginally visible because of the industrial exposure increment. As far as haemoglobin adducts are concerned, the present data confirm that, in addition to the acrylonitrile adduct *N*-(cyanoethyl)-valine (Thier et al. 1999, 2000) and to the methyl- adduct *N*-methyl-valine (Thier et al. 2001), the 4-ADP adduct may serve as a sensitive indicator of tobacco smoke exposure.

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